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1 ***In vitro* investigations on the effects of semi-synthetic, sulphated carbohydrates on the**
2 **immune status of cultured common carp (*Cyprinus carpio*) leucocytes.**

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16 Key words: Immunostimulants; Beta-glucan; Carbohydrates modification; Immune
17 responses; Innate immunity; Cytokines; *Cyprinus carpio*.

18
19 **Highlights**

- 20
21 • Modification to methyl hydroxyethyl cellulose produced a novel carbohydrate
22 (MHCS).
23 • Modified glucan (MHCS) exhibited consistent biological activities.
24 • MHCS induced strong respiratory burst in leucocytes without impacting viability.
25 • MHCS induce the expression of inflammation-related genes in carp immune cells.
26 • MHCS enhanced the effect of Poly I:C on carp leucocytes.

27
28 **Abstract**

29
30 The rapid emergence of drug resistance, unfavourable immunosuppression and mounting
31 evidence to suggest the deleterious accumulation of drug breakdown residues within animal
32 tissues has driven a strong desire to move away from these current methods of disease
33 control. Some natural products such as β -glucan, which are extracted from, for example,
34 plants and fungi, are able to modulate the immune system and increase protection against
35 diseases. However, these products are heterogeneous and their effects can be variable thus
36 limiting their applicability and reliability. Carbohydrates were modified via chemical
37 sulphation and these semi-synthetic, sulphated carbohydrates analysed for their
38 immunological activity utilising carp pronephric cells and a carp leucocyte cell line (CLC). A
39 sulphated $\beta(1,4)$ -glucan, methyl hydroxyethyl cellulose sulphate (MHCS), demonstrated a
40 stimulatory effect on fish immune cells. MHCS induced a range of bioactive effects in carp
41 leucocyte cells whilst not affecting cell viability when cells were exposed for 24h at
42 concentrations of 1-150 μgml^{-1} . MHCS stimulated the innate immune system where a
43 significant increase in respiratory burst activity was observed at concentrations 25-250 μgml^{-1}
44 in comparison to control (sterile water), cellulose ether, MacroGard[®] and zymosan. Also,
45 under in mock bacterial and viral infection conditions i.e. Lipopolysaccharide (LPS) and
46 polyinosinic:polycytidylic acid (Poly(I:C)), MHCS enhanced the immune responses of
47 pronephric cells by stimulating the respiratory burst activity at concentrations 50 and 150
48 μgml^{-1} . MHCS also enhanced the expression of cytokines including interleukin 1 beta (IL1 β),

49 tumor necrosis factor alpha 1 and 2 (TNF α 1,2), interferons alpha 2 (IFN α 2) and inducible
50 nitric oxide synthase (iNOS) in carp pronephric cells. It is proposed that this new semi-
51 synthetic carbohydrate is a potential candidate for the development of a new generation of
52 immunostimulants and adjuvants for use in vaccination strategies in aquaculture.

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53 1. Introduction

54
55 Aquaculture is a rapidly developing sector in animal production; however infectious
56 diseases remain a major obstacle to the expansion of this industry. The widespread use of
57 antibiotics and chemotherapeutics has conventionally been deployed to ameliorate
58 undesirable infections. The rapid emergence of drug resistance, unfavourable
59 immunosuppression and mounting evidence to suggest the deleterious accumulation of drug
60 breakdown residues within animal tissues such as Microcystin-LR in Yellow Perch (*Perca*
61 *Flavescens*) (Dyble et al., 2011), has driven a strong desire to move away from these current
62 methods of disease control, thus alleviating their associated negative environmental and
63 potential health-associated impacts (Anderson, 1992). Routine vaccination, used to strengthen
64 the immune system in the fish and protect against infection, has emerged as an effective and
65 economically viable means of disease control although to-date, many infectious diseases of
66 worldwide importance are not currently preventable by vaccination programmes.

67 Recent research has concentrated on the development of natural disease control strategies,
68 which bolster the immune system of the fish through the administration of
69 immunomodulatory compounds (Maudling, 2006). Such immunomodulators are able to
70 regulate the immune system through their innate ability to stimulate and/or suppress various,
71 distinct components within the immune system of fish (Zapata et al., 1997). An example of
72 an immunomodulatory agent that has found widespread use in aquaculture is the
73 carbohydrate immunostimulant, $\beta(1,3 \pm 1,6)$ -glucan. These naturally derived carbohydrates
74 act by enhancing both the innate and adaptive immune system. Administration is normally
75 carried out through injection, although the use of less invasive immersion bath technologies
76 and/or formulation within fish feeds are the preferred methods of deployment (Herman,
77 1970).

78 Beta-glucan, a polysaccharide composed of repeating β -D-glucose monomers linked by
79 1,3 and 1,6 glycosidic bonds, is obtained from the cell wall of many microorganisms, cereals,
80 fungi, seaweed and algae. The most frequent sources are baker's and brewer's yeasts
81 *Saccharomyces cerevisiae* (Novak and Vetvicka, 2008, Petravić-tominac et al., 2010), which
82 have been investigated in both laboratory and clinical studies.

83 Natural β -glucans exhibit a variety of immune-related activities that are dependent on
84 underlying composition and fine structure, molecular weight, linkage type and branching
85 pattern; these also dictate the varying solubility of this class of molecules (Li et al., 2013).
86 This structural complexity and their inherent batch-to-batch variability leads to difficulties in
87 predicting the immunoactivity profile of β -glucans, which are also capable of provoking
88 undesirable side-effects when such heterogeneous immunomodulators are utilised as feed
89 supplements or adjuvants. Both the Centre for Veterinary Medicine (CVM) and the Food and
90 Drug Administration (FDA) in the United States have implemented several requirements,
91 which must be adhered to for regulatory approval of aquaculture feeds and drugs. These
92 requirements includes general examinations i.e. the determination of physicochemical
93 parameters (e.g. pH), homogeneity, the presence of foreign particles, or microbial
94 contamination (e.g. *Salmonella*, *Coliform* and *Vibrio*) and the detection of heavy metal
95 contaminants (e.g. lead, cadmium and mercury). And special examination parameters include
96 both qualitative and quantitative testing to elucidate an in-depth structural analysis of the
97 products. Therefore, it is desirable that any immunomodulatory agent, which is to be
98 formulated with animal feed, or deployed as a drug, has an identifiable structure that can be
99 reproduced in a facile manner, free from batch-to-batch variation.

100 Natural polysaccharides can be augmented by sulphation, thereby producing physically
101 and chemically modified polymers to assist the development of new biomaterials. The

102 aforementioned modifications may bestow important therapeutic and biological activities,
103 examples being the modulation of coagulation by sulphated oat β -glucan (Chang et al., 2006),
104 the antitumor potential of sulphated α -(1-3)-D-glucan obtained from the fruiting bodies of
105 *Ganoderma lucidum* (Zhang et al., 2000) and microbial invasion blocking, e.g. HIV with
106 sulphated curdlans (Yoshida et al., 1995), or sulphated *Konjac glucomannan* (Bo et al.,
107 2013). These bioactive carbohydrates are dependent upon the presence of sulphate groups
108 that play an important role in a variety of regulatory and modulatory processes, combined
109 with binding and recognition events between specific carbohydrate structures (negatively
110 charged sulphated group) and their protein partners (usually positively charged peptide
111 sequences). Binding is affected by the presence of suitable polar groups, the degree of
112 sulphation, associated cations, molecular weight and the chain conformation of the
113 polysaccharides (Toida et al., 2003, Bo et al., 2013).

114 The mechanism of action of sulphated carbohydrates in carp is currently unknown but, it
115 seems likely that at least in part, it is the result of the ability of sulphated carbohydrates to
116 mimic the endogenous glycosaminoglycan (GAG) polysaccharides. The GAGs are naturally
117 occurring, sulphated carbohydrates that are well conserved through evolution, with GAG
118 species identified in bacteria, fish, reptiles, molluscs, arachnids, insects and mammals (Volpi,
119 2005). Numerous, distinct roles for the GAG class of polysaccharides have been elucidated in
120 recognition, binding, regulation and modulation of many proteins, including those involved in
121 the hosts immune system (Skidmore et al., 2008, Rudd et al., 2010a). Indeed, growing
122 evidence suggests that the physiological role of heparin, a pharmaceutical anticoagulant, is
123 most likely to be in an immunomodulatory capacity and not the antithrombotic capacity for
124 which it is best known. Previous work by the authors has demonstrated that semi-synthetic,
125 sulphated carbohydrates can mimic the biological activities of the glycosaminoglycan (GAG)
126 class of naturally occurring, sulphated carbohydrates (Rudd et al., 2010b). This study has pre-
127 screened an extant library of sulphated carbohydrates, acting as GAG analogues, which have
128 been shown previously to possess favourable bioactivity in biological systems known to be
129 modulated by GAGs (Rudd et al., 2010b, Boyle et al., 2017, Skidmore et al., 2017). The
130 constituent sulphated glycans of the library resource cover a wide spread of chemically
131 diverse sequence-space (including chemically sulphated β -glucans), and this study as
132 identified a sulphated β (1,4)-glucan, methyl hydroxyethyl cellulose sulphate (MHCS), as a
133 potential candidate for favourable immunomodulation in aquaculture with future potential as
134 an adjuvant. Furthermore, the negligible cytotoxicity of this carbohydrate-based candidate
135 has been demonstrated, along with its favourable immunostimulatory potential within the
136 inflammatory response of relevant fish cells.

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140 2. Materials and Methods

141 2.1 Preparation of MacroGard[®], zymosan and cellulose ether immunostimulants

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144 Concentrations of MacroGard[®] (Biorigin; a bakers' yeast extract containing 60% of β -
145 1,3: β -1,6 glucan), zymosan (Sigma, Z4250; a β -1,3 linked glucan) and cellulose ether
146 (Tylose) (Sigma 93802; a linear, β -1,4 linked glucan) were prepared as described by Vera-
147 Jimenez et al. (2013). Owing to the innate insolubilities of the parental material appropriate
148 concentrations of the aforementioned carbohydrates were made-up in sterile-filtered water
149 (Sigma, W3500) and sonicated twice for 30 s (Sonics Vibra-cell, power setting 6). To ensure
150 sterility, the stock solution was heated to 80°C for 20 min prior to incubation at $19 \pm 1^\circ\text{C}$.

151 2.2 Preparation of pathogen associated molecular patterns (PAMPs)

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153
154 LPS from *E.coli* 0111:B4 strain (Invitrogen) and Poly(I:C) (a synthetic analogue of
155 dsRNA; Invitrogen), were prepared as per the manufacturer's instructions and diluted to the
156 required concentration with sterile-filtered water.

157 2.3 Preparation of semi-synthetic sulphated carbohydrate based immunostimulants

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159
160 Semi-synthetic carbohydrates were sulphated by a modified version of the chlorosulfonic
161 acid (CSA) sulphation protocol as described by Yoshida et al. (1995), which modifies
162 amenable hydroxyls. Briefly, the powdered precursor carbohydrates (0.5 g) were dissolved in
163 ice-cooled 5 ml dimethylformamide (Sigma), 10 ml pyridine (VWR) and 1 ml chlorosulfonic
164 acid (Sigma). The mixtures were heated to 95 °C for 2 h, cooled over ice and slowly
165 neutralized with sodium hydroxide (50% w/v; VWR). Ethanol precipitations were performed
166 in saturated sodium acetate (Sigma), overnight at 4 °C. Precipitates were dissolved in
167 deionized water and dialysed (Mw cut off > 7 kDa; VWR) for 72 h against HPLC grade
168 water (VWR). The sulphated carbohydrates were lyophilised and resuspended in sterile
169 filtered water at appropriate concentrations prior to use. Confirmation of sulphation and the
170 degree of sulphation of bioactive saccharides was achieved using sodium rhodizonate, based
171 on the method described by Terho and Hartiala (1971) [supplementary data, Figure 1].
172 Further evidence of precursor modification was obtained using Attenuated Total Reflection
173 Fourier Transform Infrared (FTIR-ATR) spectroscopy [supplementary data, Figure 2].

174 2.4 *In vitro* Carp leucocytes cell line culture.

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176
177 Carp leucocytes cell lines (CLCs) is a permanent cell line established from peripheral
178 blood mononuclear cells obtained from a normal, non-leukemic, non-virally infected
179 common carp. CLC morphology characterise with an epithelial like shape and exhibiting
180 functions similar to monocytes and macrophages including adherence to plastic and
181 phagocytosis of iron particles (Faisal and Ahne, 1990). CLC exhibited respiratory burst
182 activity after stimulation with Phytohaemagglutinin (PHA) and LPS and this was similar to
183 head kidney macrophages responses (Koumans-van Diepen et al., 1994). CLC suitability for
184 studies on macrophage activation, and as *in vitro* model to study the immune responses of
185 fish was concluded in both studies by Weyts et al. (1997) and Vidal et al. (2009). In addition,
186 the CLC stimulate leukocyte proliferation by producing interleukin-1like factors (Weyts et
187 al., 1997). The CLC line were kindly provided by Wageningen University, The Netherlands
188 and were grown at 27°C and 5% CO₂ in L-glutamine free RPMI (Sigma) modified with 5%

189 (v/v) foetal bovine serum (Sigma), 2.5% (v/v) heat-inactivated pooled carp serum, 50 U/ml
190 penicillin-G, and 50 mgml⁻¹ streptomycin (Sigma, P4458); this modified medium referred to
191 hereafter as CLC RPMI⁺. Cultures were split (1:3 v/v) when reaching 80% confluence;
192 culture medium was replenished every 3 days. The trypan blue exclusion assay (Howard and
193 Pesch, 1968, Hauton and Smith, 2004) was used to determine cell viability and only cell
194 suspensions with at least 95% viability were used for experimentation.

195

196 **2.5 Preparation of pronephric cell suspension**

197

198 Common carp, *Cyprinus carpio* (Fair Fisheries, Shropshire, UK), were maintained in
199 black plastic tanks with recirculating water at 15 °C and pH 7, and kept on a 12 h:12 h, light:
200 dark cycle. Approximately 25 fish were kept in each tank and were fed daily on commercial
201 pelleted feed that lacked an immunostimulant additive (Tetra GmbH, Germany). The
202 pronephros was removed from 5 carps (89.6 ± 12.4 g), which had been sacrificed previously
203 by a lethal dose (~ 0.2% v/v) of 2-phenoxyethanol (Sigma). Blood was collected from the
204 caudal vein before dissection. The isolated pronephros was placed in modified RPMI medium
205 on ice, under sterile conditions and a cell suspension was prepared using a modification of the
206 procedure described by Kemenade et al. (1994). Briefly, pronephros tissue was disrupted
207 gently through a sterile cell strainer with 100 µm pore diameter (BD Falcon) in 1 ml of
208 modified RPMI medium that comprised RPMI supplemented with 0.3 gL⁻¹ L-glutamine
209 (Sigma), 0.5% (v/v) sterile water, 0.05% (v/v) heat-inactivated pooled carp serum, penicillin
210 (50 U/ml), and streptomycin (50 µgml⁻¹) (Sigma); this modified medium referred to hereafter
211 as RPMI⁺. A non-continuous Percoll gradient (Sigma) was used to isolate leucocytes, which
212 were collected at the interphase between densities 1.02 gml⁻¹ and 1.08 gml⁻¹, washed three
213 times with RPMI⁺ and centrifuged at 4 °C (800 g; 10 min).

214

215 **2.6 MTT cell proliferation assay**

216

217 Potential toxic effects on cell proliferation of administered semisynthetic, sulphated
218 carbohydrates were determined utilising the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-
219 diphenyl tetrazolium bromide (MTT) assay and compared to other immunostimulants.
220 Briefly, a serial dilution of the test carbohydrate (1-150 µgml⁻¹) was prepared and added to
221 CLCs (2 × 10⁴ cells in 100 µl per well) in a 96 multiwell plate (Sarstedt). A negative control
222 comprising 5 µl sterile water and positive controls comprising 50 µgml⁻¹ of MacroGard[®] and
223 zymosan were also included. After 24 h the MTT assay was performed by adding 10 µl per
224 well of the MTT solution (5 mg of MTT; Sigma) dissolved in 1 ml of PBS (Life
225 technologies). The plate was then incubated for 4 h at 27 °C with 5% CO₂, the supernatant
226 discarded, the cells solubilised with 100 µl of dimethyl sulfoxide (Fisher) and the relative
227 levels of proliferation measured indirectly by spectrophotometry at a λ_{abs} of 540 nm (Ferrari
228 et al., 1990).

229

230 **2.7 Trypan blue cell viable cell count assay**

231

232 Trypan blue cell viability assays were conducted with and without the addition of
233 semisynthetic, sulphated carbohydrate addition and other relevant immunostimulants. CLC
234 lines (2 × 10⁵ cells in 1 ml of CLC RPMI⁺ medium) were distributed in 24 wells plates and
235 stimulated by adding 15 µl per well of carbohydrate sample at 1, 2.5, 50, 150 µgml⁻¹. A
236 negative control comprising 15 µl per well of sterile water and a positive control comprising
237 15 µl per well of MacroGard[®] at a concentration of 50 µgml⁻¹ were also included. After 24 h

238 incubation at 27°C and 5% CO₂, the culture medium was aspirated, and the cells washed once
239 with pre-warmed CLC RPMI⁺ medium. Cells were then detached by adding 250 µl per well
240 of 0.25x Trypsin-EDTA (Sigma) for 1 min, washed 3 times with 500 µl of CLC RPMI⁺
241 medium, centrifuged at 750 g for 5 min at 19 ± 1°C and the supernatant discarded. The cell
242 pellet was re-suspended in 500 µl of fresh CLC RPMI⁺ and viable cells were determined
243 using trypan blue solution (0.4% w/v).

244

245 **2.8 Respiratory burst activity screen**

246

247 The NBT assay was performed as described by Vera-Jimenez et al. (2013) to determine
248 respiratory burst activity. Briefly, CLCs were placed in CLC RPMI⁺ with the exception that
249 the RPMI medium used was free of phenol red (Sigma). A cell monolayer was formed at the
250 bottom of the flat 96 multiwell plate (Sarstedt), which were incubated at 27°C in 5% CO₂ for
251 2-3 h. The supernatants were discarded, the cells washed with phenol red free Hank's
252 balanced salt solution (HBSS) (Sigma) and 160 µl of CLC RPMI⁺ containing NBT at 1
253 mgml⁻¹ (Sigma) was added to each well. The respiratory burst activity was induced by adding
254 5 µl of increasing concentrations of test solutions containing either MacroGard[®] (1-150 µgml⁻¹)
255 ¹), zymozan (1-150 µgml⁻¹), methyl hydroxyethyl cellulose (2.5-250 µgml⁻¹) or the sulphated
256 derivative of the latter (MHCS; 1-250 µgml⁻¹). Poly(I:C) (100 µgml⁻¹) and LPS (50 µgml⁻¹)
257 were also assayed as non-carbohydrate controls. After incubation at 27°C in 5% CO₂, the
258 supernatants were decanted, the cells fixed with ice cooled methanol (100 µl, 3 min) and the
259 plates left to air dry. The membranes of the phagocytic cells were solubilised with 120 µl
260 KOH (2 M) and 140 µl of DMSO added to solubilise the blue formazan. The reduction of
261 NBT was measured spectrophotometrically at λ_{abs} of 620 nm.

262

263 **2.9 Carbohydrate based modulation of immune-associated gene expression**

264

265 Pronephros cells prepared from 3 fish individually at 4 × 10⁶ cells per well, in 2 ml were
266 cultured in 6 well plates (Sigma) and exposed to 60 µl of either MacroGard[®] (50 µgml⁻¹),
267 LPS (50 µgml⁻¹), Poly(I:C) (100 µgml⁻¹) and MHCS (50, 150 µgml⁻¹). Cells were
268 subsequently harvested after 6, 12 or 24 h incubation at 27°C with 5% CO₂, using 0.25x
269 Trypsin-EDTA solution, washed 3 times with pre-warmed PBS and collected by
270 centrifugation (800 g for 10 min at 4°C).

271

272 RNA was extracted from cell pellets using an RNeasy kit (Qiagen) and cDNA formed
273 using the M-MLV RT kit (Invitrogen). Briefly, a mixture of 500 ng of RNA sample, 1 µl of
274 50 µM random hexamers, 1 µl 10 mM dNTPs and 4.5 µl of DEPC water were heated at 65°C
275 for 5 min before immediate cooling on ice. After a brief centrifugation, 4 µl of 5X First-
276 Strand buffer, 2 µl of 0.1 M DTT and 1 µl of RNaseOUT[™] recombinant ribonuclease
277 inhibitor (40 units/ µl; Invitrogen) were added and mixed gently. The mixture was heated at
278 37°C for 2 min and 1 µl (200 units) of M-MLV RT enzyme added before mixing thoroughly.
279 The reverse transcriptase reactions were carried out after the samples were incubated at 25°C
280 for 10 min followed by 50 min at 37°C, then enzymes were heat inactivated by incubation at
281 70°C for 15 min. Samples were diluted 1:10 (v/v) with DEPC treated water and stored at -20

282

283 °C. Carp specific primers were used to determine the expression levels of IL1β, TNFα1,
284 TNFα2, iNOS and IFNα2 genes (Table 1); the ribosomal 40S gene was used as a
285 housekeeping gene (Miest et al., 2012). The PCR reactions were carried out in 96 well PCR
286 plates (Applied Biosystems, MicroAmp[®]). Briefly, 2 µl of cDNA were added to 10 µl
SensiFAST, (Bioline, BIO-92020), 0.8 µl of 10 µM forward and reverse primers respectively

287 (reaction mixture final concentration equal to 400 nM) and made up to 20 μ l with DEPC
288 treated water (Invitrogen). The PCR plate was centrifuged (660 g, 4 min) (Boeco) prior to
289 analysis. A cycling procedure was carried out with 1 cycle at 95°C for 2 min, 40 cycles at
290 95°C for 5 sec and 30 sec at 62°C. The melting curves of the PCR products were determined
291 after each run between 60 and 95°C. The $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001) of
292 targeted genes were normalised against the reference gene 40S, and the x-fold change
293 calculated relative to the control group for each time point.

294

295 **2.10 Statistical analysis**

296

297 Statistical analyses were carried out using GraphPad Prism 5 and SPSS 21, with all data
298 presented as the mean \pm standard error. Data were tested for normality and equal distribution
299 of variance. A one-way analysis of variance (ANOVA) and Tukey's post-hoc test were
300 performed on the bioactivity data of MHCS with regard to cell proliferation, viability and
301 ROS production. A two-way analysis of variance (ANOVA) and post-hoc Bonferroni's
302 multiple comparisons test were used in the comparison experiments between semi-synthetic
303 sulphated carbohydrates and different immunostimulants. Gene expression data were
304 normalised using a Log10 transformation prior to a two-way ANOVA prior to conducting
305 Bonferroni post-hoc test analyses. Significance was defined as $p \leq 0.05$.

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3. Results

3.1 The effect of MHCS on cell viability & proliferation

The MTT cell proliferation assay demonstrated that the MHCS was not cytotoxic to the CLC line over the concentration range screened. Indeed, at concentrations of 1 and 2.5 μgml^{-1} MHCS promoted a significant increase ($p \leq 0.0011$, $p \leq 0.002$, respectively) in cell proliferation (Figure 1). The trypan blue exclusion assay confirmed this observation and supported the significant increase ($p \leq 0.026$) in cell growth after treatment to 2.5 μgml^{-1} of MHCS (Figure 2).

3.2 MHCS induced respiratory burst activity

Statistical analysis revealed a clear trend and significant increase in the respiratory burst activity of CLC cells after treatment with MHCS ($F = 68.668$, $p < 0.0001$). The concentration dependency effect of MHCS increased significantly ($p \leq 0.022$) at 25, ($p < 0.0001$) 50, 75, 150 μgml^{-1} and at the latter concentration, induced an increase in respiratory burst activity 4.5x greater than that of the control, 5 μl sterile water (Figure 3).

The respiratory burst activity in CLCs after treatment to methyl hydroxyethyl cellulose and MHCS confirmed that the modification of the polysaccharide by chemical sulphation conferred favourable bioactivity on the parental molecule (Figure 4). The levels of cell respiratory burst activity were significantly dependent upon the carbohydrate type ($F = 26.24$, $p < 0.0001$) and their concentrations ($F = 38.23$, $p < 0.0001$). The Bonferroni test revealed significant differences ($p < 0.001$) between the modified and precursor carbohydrates at concentrations between 25-250 μgml^{-1} , suggesting that the addition of sulphate moieties affects the biological activity of the cellulose ether. MHCS promoted the respiratory burst activity of CLC cells ($p < 0.0001$) in comparison to the control. The results further support the data presented in Figure 3.

Experiments on the respiratory burst activity of CLC line was extended to include MacroGard[®] and zymosan in comparison with the MHCS at concentrations in the range of 1-150 μgml^{-1} for 24 h. The assay demonstrated that the type of carbohydrate ($F = 834.8$, $p < 0.0001$), the concentration ($F = 211.4$, $p < 0.0001$) and the interaction of these two factors ($F = 172.3$, $p < 0.0001$) significantly influence the reactive oxygen species induced in CLC cells (Figure 5). The effect of MHCS was significant in comparison to MacroGard[®] and zymosan at concentrations of 25, 50, 100 and 150 μgml^{-1} (all at $p < 0.0001$). The chemically modified carbohydrate MHCS, stimulated CLC respiratory burst activity and presented a significant increase at concentrations ≥ 25 μgml^{-1} when compared against control (5 μl sterile water) ($p < 0.0001$).

3.3 Pathogen associated molecular patterns (PAMPs) and MHCS in pronephric cells

In order to determine whether MHCS treatment increased the immune response of pronephric cells under mock infection conditions, the level of reactive oxygen species (ROS) and effect on cell viability were determined post treatment to LPS and Poly(I:C) (Figure 6 and 7). ROS production in cells was significantly augmented ($p < 0.0001$) at 50 and 150 μgml^{-1} of MHCS irrespective of LPS treatment, when compared to the control (5 μl sterile water) (Figure 6 A). The treatment of pronephric cells with MHCS did not perturb cell viability at both concentrations either alone, or in combination with LPS (Figure 6 B). Treatment with MacroGard[®] at 50 and 150 μgml^{-1} alone, or with LPS, did not affect the

385 production of ROS. However, a significant decrease in cell viability was observed, when
386 compared to control, for cells treated with MacroGard[®] at 50 μgml^{-1} with LPS ($p < 0.0001$),
387 MacroGard[®] alone at 150 μgml^{-1} ($p = 0.024$) and MacroGard[®] at 150 μgml^{-1} with LPS ($p =$
388 0.004). Significant differences ($p = 0.015$) between MacroGard[®] at 50 μgml^{-1} and
389 MacroGard[®] supplemented with LPS were also observed (Figure 6 B).

390 Treatment with Poly(I:C) induced a significant increase in ROS production alone ($p =$
391 0.0002) and in combination with either MacroGard[®] or MHCS ($p < 0.0001$) when compared
392 to the control (5 μl sterile water) (Figure 7 A). The production of ROS in response to
393 MacroGard[®] treatment at 50 and 150 μgml^{-1} had no effect, however when exposed with
394 Poly(I:C), the ROS levels increased and were significantly different ($p = 0.005$, $p < 0.0001$
395 respectively) to MacroGard[®] alone. Both MHCS alone, and in the presence of Poly(I:C),
396 induced highly significant increases in ROS production in comparison to the control ($p <$
397 0.0001 ; Figure 7 A). Interestingly, stimulation with MHCS at both concentrations induced a
398 significant increase in ROS production of Poly(I:C) treated cells when compared to their
399 respective concentrations of MHCS without Poly(I:C) and Poly(I:C) alone (Figure 7 A). The
400 MTT assay shows no significant differences in cell proliferation, and hence viability in all
401 treatment groups in comparison to the relevant control (Figure 7 B).

402

403 3.4 Immune gene expression in pronephric cells

404

405 The expression levels of the inflammatory cytokines IL1 β and TNF α 1 were increased
406 significantly post treatment with MHCS (Figure 8). IL1 β expression was up-regulated
407 significantly ($p < 0.0001$) after 6 h post treatment at both MHCS concentrations and
408 remained up-regulated after 12 ($p = 0.01$) and 24 h ($p = 0.03$) post treatment to MHCS at a
409 concentration of 150 μgml^{-1} . While TNF α 1 expression increased significantly after 6 h post
410 treatment with both MHCS at 50 μgml^{-1} ($p = 0.015$) and 150 μgml^{-1} ($p = 0.0001$). Only
411 MHCS at 150 μgml^{-1} affected the TNF α 1 expression at 12 h ($p = 0.012$) and 24 h ($p = 0.008$)
412 post treatment. However, the expression of TNF α 2 was up-regulated significantly only at 6 h
413 post treatment with MHCS at 50 μgml^{-1} ($p = 0.046$) and 150 μgml^{-1} ($p < 0.0001$).

414 MacroGard[®] induced significant IL1 β expression after 6, 12 and 24 h post treatment ($p =$
415 0.031 , 0.002 , 0.021 respectively). Furthermore, a temporal response was observed in the
416 expression of TNF α 1 and TNF α 2 at 6, 12 and 24 h post treatment with MacroGard[®]. The up-
417 regulation was highly significant at all-time points ($p \leq 0.0001$) except for 6 h post treatment,
418 where the expression of only TNF α 1 was significant at $p = 0.002$. In contrast, elevated
419 expression levels of IL1 β , post treatment with LPS, were only induced significantly ($p =$
420 0.007) after 6 h post treatment. Furthermore, iNOS expression levels were increased
421 significantly after 6 h treatment to MHCS, at concentrations of 50 and 150 μgml^{-1} ,
422 respectively ($p = 0.019$, $p < 0.0001$). LPS also induced significant ($p = 0.017$) iNOS
423 expression at 6 h post treatment. In comparison, MacroGard[®] induced a late significant ($p <$
424 0.0001) iNOS expression at 12 and 24 h post treatment.

425 Poly(I:C) at 100 μgml^{-1} had no effect on either the inflammatory cytokines studied nor
426 upon iNOS expression (Figure 8). However, the expression levels of IFN α 2 were up-
427 regulated significantly at all-time points studied after treatment to Poly(I:C) at 100 μgml^{-1} (p
428 < 0.0001 ; Figure 8).

429

430

4. Discussion

The results show that a modified carbohydrate polymer such as MHCS can be generated with important biological activates and immunostimulatory effects in carp. Although, there are several methods to induce glucan modifications, sulphation has the strongest effects on biological function (Han et al., 2008). Previous studies in other non-fish systems have suggested that the sulphation process may alter the chemical and biological properties of glucans. For example, sulphated *Konjac glucomannan*, induces a high anti-HIV activity in the MT-4 cell line similar to the acquired immune deficiency syndrome (AIDS) drug (Bo et al., 2013). In another investigation, the presence of a sulphate group on the lentinan structure caused significant increases in antioxidant activity (Feng et al., 2010). Furthermore, rice bran β -glucan that was subjected to sulphation had a significant difference to the native oat glucan in molecular weight, solubility, viscosity and exhibited anticoagulant activity in rat blood (Chang et al., 2006).

The immune system recognises immunostimulants by the presence of pathogen recognition receptors (PRRs) that are present on the outer membrane of the immune cells. This recognition leads to activation of the immune cell and enhancement of their responses, which usually comprises an increase in their bactericidal activities, including the stimulation of phagocytosis, leucocyte migration and the production of cytokines (e.g. IL-1, TNF α), nitric oxide (NO) and reactive oxygen species (Sakai, 1999). In mammals, phagocytosis, believed to be the uptake mechanism of β -glucan, leads to their antimicrobial activity by the induction of reactive oxygen, and nitrogen species production and lytic enzymes in phagosomes (Goodridge et al., 2009). Several characteristic phagocytosis receptors on carp macrophage, including the complement receptor 3 (CR3), Scavenger Receptors (SRs) and C-type lectin receptor (CLR) superfamilies, and sensing receptors such as TLR2 (Petit and Wiegertjes, 2016) have been recognised as detecting β -glucan.

In addition, several studies have also highlighted the dose effects of β -glucans on cell cytotoxicity/ viability, for example in an investigation carried out on common carp, a significant increase in apoptosis occurred when pronephric cells were stimulated with β -glucans at concentrations 500 μgml^{-1} and higher for 6 h incubation (Miest and Hoole, 2015).

The MHCS was able to trigger several bioactive mechanisms i.e. cell viability, increase leucocyte number and respiratory burst activity. Interestingly, MHCS promoted a rapid increase in respiratory burst activity, which started at 25 μgml^{-1} concentrations and reached more than four and half times higher than the control at 150 μgml^{-1} . This linear dose/effect relationship is unusual for an immunostimulant because, often, the effect occurs at certain intermediate concentrations and disappears, or even becomes toxic at high concentrations (Kum and Sekkin, 2011). This steep increase in respiratory burst activity did not cause exhaustion to the immune cells as supported by the viability and cell count assays. This was a promising result, encouraging the debate whether the modification (sulphation) was the reason behind this biological effect. In a previous study it was shown that the soluble form of β (1-3)-glucan had some protective properties against infection in mice. The results of that study showed an increase in neutrophils in blood stream, enhancement in bone marrow proliferation and *in vitro* phagocytic activity to *E. coli* bacteria (Tzianabos, 2000).

The biological activities of modified carbohydrate MHCS were compared to the native source (cellulose ether) and different β -glucans. Interestingly, cellulose ether, zymosan and MacroGard[®] had no significant effects on CLCs line respiratory burst activity, while MHCS induced a significant increase in comparison to control and previous carbohydrates at a concentration equal to and higher than 25 μgml^{-1} . These rapid responses to MHCS carbohydrate by fish leucocytes might be due to carbohydrate-protein interactions. Sulphation

480 provides polysaccharides negative charges at the sulphate groups, which may be interacting
481 with positive peptide sequence of proteins (Chang et al., 2006). This is in line with the many
482 biological activities, which have been shown in heparin sulphation such as regulation of
483 cellular growth and proliferation, cell adhesion, blood coagulation, cell surface binding of
484 proteins, viral invasion, and tumour metastasis (Rabenstein, 2002).

485 Glucan solubility not only depends on the degree of polymerisation and branching,
486 but also on chemical derivations including sulphation (Chang et al., 2006). The degree of
487 substitution (DS) indicates the average number of sulphate groups attached to a glucose unit.
488 Sodium rhodizonate assay was used to determine the DS of the sulphated derivative MHCS
489 and was determined as $1.74 \text{ moles}\mu\text{g}^{-1}$ of disaccharides. This demonstrates that sulphation
490 was sufficient to induce important biological activities without causing detrimental side-
491 effects e.g. cell death. When the degree of sulphation is high, there is an increased chance of
492 undesirable anticoagulant activities for modified carbohydrates, e.g. highly sulphated
493 carbohydrates such as dextran sulphate (degree of sulphation $5.25 \text{ moles}\mu\text{g}^{-1}$ of
494 disaccharides) has a high anticoagulant activity in human blood (Yoshida et al., 1995).
495 Although the correlation between sulphation levels and anticoagulant potential is complex it
496 should be noted that teleost fish coagulation system is fundamentally similar to that of
497 mammals, in spite of the significant evolutionary distance between these groups (Tavares-
498 Dias and Oliveira, 2009). However, blood coagulation time in fish is shorter in comparison to
499 mammalian and depend on fish species (Wolf, 1959, Doolittle, 1962, Smit and Schoonbee,
500 1988).

501 It is possible that MHCS may form the basis of a new carbohydrate adjuvant in
502 vaccine production. Therefore, before applying the MHCS to *in vivo* conditions, the effect of
503 MHCS under mimicked infection conditions using PAMP immunostimulants were
504 determined in carp pronephric cells. The results revealed that MHCS induced a significant
505 increase in respiratory burst activity regardless of the LPS availability. LPS did not induce
506 respiratory burst activity in pronephric cells when exposed alone, and had no additional
507 effects on cells when exposed with MacroGard[®] or MHCS. This is despite the ability of LPS
508 to stimulate the non-specific and specific immune responses in fish, and its recognitions by
509 toll-like receptor 2 and 4 (TLR2, TLR4) in immune cells that induce a signalling cascade
510 leading to the activation of NF- κ B and the production of proinflammatory cytokines (Swain
511 et al., 2008). However, responses to LPS can vary depending upon its source (Bich Hang et
512 al., 2013), and its effects on macrophage respiratory burst activity appeared to be dose and
513 incubation time dependent (Solem et al., 1995). Nayak et al. (2011) reported the high
514 variability of the external polysaccharide region of LPS, and the differences in potency and
515 spectrum of action of the lipid A components in many Gram-negative bacteria. This might be
516 the explanation of the difference in the LPS effects on fish immunity, for example Watzke et
517 al. (2007) noted the low sensitivity of zebrafish immune cells to LPS from *Escherichia coli* in
518 comparison to *Edwardsiella tarda*. In addition, LPS from *Aeromonas hydrophila* stimulated
519 carp (*Cyprinus carpio*) immune responses and enhanced fish protection against another
520 aromonad *Aeromonas hydrophila* infection when exposed via intraperitoneal injection and
521 bathing (Selvaraj et al., 2009).

522 Both MHCS and Poly(I:C) induced significant increases of the respiratory burst
523 activity when exposed alone. Also, Poly(I:C) boosted the respiratory burst activity of both
524 MacroGard[®] and MHCS treated cells. This might be due to the different uptake pathways of
525 Poly(I:C), MacroGard[®] and MHCS. It is well established that Poly(I:C), which is used as a
526 synthetic viral dsRNA analogue, induce IRF-3 (interferon regulatory factor-3) activation via
527 the TLR3 (is an endosomal PRR of the innate immunity) and the synthesis of interferon-
528 stimulated genes that restrict virus replication (Wang et al., 2009). While the main receptors

529 that are associated with glucans comprise C-type lectin receptor Dectin-1, complement
530 receptor 3 (CR3), scavenger receptors (SRs), glycolipids or Carbohydrate Binding Module
531 (CBM) (Legentil et al., 2015, Meena et al., 2012). Poly(I:C) recognition pathway was
532 observed in pronephric cells of carp, where only Poly(I:C) induced significant up regulation
533 of IFN α 2 expression. IFN-alpha is a type I IFN that has a major role in the first line of
534 defence against viruses. In mammals, the type I IFN antiviral effect is binding to the IFN- α / β -
535 receptor, which triggers the JAK-STAT signal transduction pathway resulting in expression
536 of Mx and other antiviral proteins (Robertsen, 2006). The difference in Poly(I:C) and MHCS
537 uptake pathways might be the reason for the boosting of the immune cells responses more
538 than when they are exposed alone.

539 MHCS induced the expression of pro-inflammatory cytokines (IL1 β , TNF α 1 and
540 TNF α 2), and inducible nitric oxide synthase (iNOS) 6h post treatment and the effect was
541 dose dependant. Interestingly, the effect of MacroGard[®] was time dependant and increased
542 with incubation time. MacroGard[®] time dependency was also observed by Miest and Hoole
543 (2015) *in vitro*, where the pro-apoptotic effect was noted to be time and dose dependent only
544 with concentrations of $\geq 500 \mu\text{gml}^{-1}$ causing apoptosis in carp pronephric leucocytes.

545 The above information is evidence of the ability of MHCS to be recognised by the
546 innate immune system through pattern recognition receptors (PRRs) including Dectin-1 (β -
547 glucan receptor (β GR), mannose receptor, complement receptors CR3, Toll-like receptors 2
548 and 6 (TLRs-2/6), scavenger receptors and lactosylceramide (Gantner et al., 2003, Herre et
549 al., 2004, Chan et al., 2009, Kim et al., 2011). Glucans binding to the above receptors led to
550 activation of several pathways and triggers several protection mechanisms i.e. phagocytosis,
551 induction of pathogen killing activity, production of inflammatory cytokines and chemokines,
552 and initiate the development of adaptive immunity (Gantner et al., 2003). β -glucan is
553 involved in the enhancement of mononuclear cells and neutrophil anti-microbial activity,
554 leading to improve macrophage activity and the proliferation of both monocytes and
555 macrophages, and the production of proinflammatory molecules such as complement
556 components, interleukin (IL)-1 α / β , TNF- α , IL-2, interferon (IFN)- γ , IL-4 and IL-10 (Chan et
557 al., 2009, Kim et al., 2011, Li et al., 2013). Different glucans associated with different or
558 similar receptors on immune cells do not induce the immune response equally. For example,
559 the scavenger receptors are non-opsonic receptors that have low affinity to attach to anionic
560 β -glucans, which have been sulphated chemically or originate from natural sources (algae)
561 (Meena et al., 2012). While Dectin-1 receptor has been identified as a major receptor for β -
562 glucans on mammalian leucocytes (Herre et al., 2004). The solubility and smaller molecule
563 size of MHCS might result in a rapid engulfment and lysis by the immune cells in
564 comparison to MacroGard. The adjuvant size determines the ability and speed to diffuse
565 inside tissues and reach the target. At sizes less than 40 nm, transmission is more rapid than
566 the large size adjuvant of 100 nm that occurs in polysaccharides, which are transported from
567 the injection site by dendritic cells to the immune organs (Smith et al., 2013). The new
568 smaller size MHCS thus has potential to have an increased immunostimulant affect in fish
569 when compared to the larger size carbohydrates which are currently in use e.g. β -glucan. The
570 utilisation of this new carbohydrate in an *in vivo* system is in progress.

571

572 **5. General conclusion of the study**

573

574 The present study contributes to the development of the use of carbohydrates as
575 immunostimulants in fish. This represents the first attempt to combine synthetic biochemical
576 approaches with carbohydrate design to produce a novel carbohydrate that modulates the
577 immune system at the cellular and molecular level. The MHCS exhibited a range of bioactive

578 properties such as the non-cytotoxic effect, and production ROS in immune cells. These
579 bioactivities were associated with the sulphate group in this carbohydrate structure. Also,
580 there is the potential to use this modified carbohydrate as an adjuvant in vaccines as it was
581 able to increase the immune response in mimic infection conditions and up-regulate the
582 expression of inflammatory cytokines genes.

583 Therefore, the next step is to trial MHCS and *in vivo* conditions and evaluate the adjuvant
584 potency in vaccines against important diseases in aquaculture.

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588

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592

593 Appendix A. Supplementary data

594 Supplementary data related to this article can be found at:

595

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772 **Figure legends**

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774 **Figure 1:** CLC line viability exposed to serial dilution of modified carbohydrates. Cells at
 775 density (2×10^4 cells per well) stimulated with zymosan = Z, MacroGard[®] = M at $50 \mu\text{gml}^{-1}$
 776 and range of modified carbohydrates concentrations 1 - $150 \mu\text{gml}^{-1}$ for 24 h. Statistical
 777 analyses were performed by one-way ANOVA ($p \leq 0.05$) and the significant differences
 778 between treatments in comparison to control performed with *: $p \leq 0.05$, **: $p \leq 0.01$ and
 779 ***: $p \leq 0.001$. Data represent mean \pm SEM of 6 well replicates.

780

781 **Figure 2:** CLC line count utilised by trypan blue viability assay. Cells were exposed to serial
 782 dilution of modified carbohydrates MHCS ($1-150 \mu\text{gml}^{-1}$) and MacroGard[®] at $50 \mu\text{gml}^{-1}$
 783 concentration for 24 h. Statistic comparison was performed by one-way ANOVA and the
 784 significant differences between treatments in comparison to control performed with *: $p \leq$
 785 0.05 . Data represent mean \pm SEM of 3 well replicates of 24 well plates.

786

787 **Figure 3:** Dose dependency effect of modified carbohydrates on CLCs respiratory burst
 788 level. Cell were seeded at (2×10^4 cells per well) and stimulated with zymosan at $50 \mu\text{gml}^{-1}$ =
 789 Z, MacroGard[®] at $50 \mu\text{gml}^{-1}$ = M and range of modified carbohydrates at concentration
 790 between $1-150 \mu\text{gml}^{-1}$ for 24 h incubation. Statistical analysis one-way ANOVA and Tukey's
 791 *post hoc* analysis were used and the differences between concentrations in comparable to
 792 control (non-treated cells) performed with *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.0001$. Data
 793 represent mean \pm SEM of six well replicates.

794

795 **Figure 4:** CLCs dose responses to sulphated and non-sulphated MHCS determined by NBT
 796 assay. The cells were distributed at (2×10^4 cells per well) stimulated with a range of
 797 cellulose ether and sulphated cellulose ether (MHCS) at concentrations between $2.5-250$
 798 μgml^{-1} for 24 h. Statistic comparison was performed using two-way ANOVA and Bonferroni
 799 *post hoc* test at $p \leq 0.05$ and the significant differences between concentrations in comparison
 800 to control presented with ***: $p < 0.0001$, also the comparison between the two treatments at
 801 each concentration presented with $\Delta\Delta\Delta$: $p < 0.0001$. Data represented the mean \pm SEM of 6
 802 well replicates.

803

804 **Figure 5:** Comparison of distinct β -glucan sources and MHCS carbohydrate on CLCs
 805 phagocytic activity. Cells phagocytic activity was measured by NBT assay after cells were
 806 distributed at (2×10^4 cells per well) and simulated with either MacroGard[®], zymosan or
 807 MHCS at concentrations $1-150 \mu\text{gml}^{-1}$ for 24 h. Statistical analysis was performed by two-
 808 way ANOVA and Bonferroni *post hoc* test ($p \leq 0.05$) and the significant differences between
 809 MHCS concentrations in comparison to matched control performed with ***: $p \leq 0.0001$.
 810 Data represent mean \pm SEM of 6 well replicates.

811

812 **Figure 6:** Respiratory burst production (A) and viability (B) in carp leucocytes after
 813 treatment to LPS ($50 \mu\text{gml}^{-1}$), MacroGard[®] (50 or $150 \mu\text{gml}^{-1}$) and MHCS (50 or $150 \mu\text{gml}^{-1}$)
 814 both exposed individually and in combination with LPS. Statistical analysis one-way
 815 ANOVA and Tukey's *post hoc* analysis were used and the differences between
 816 concentrations in comparable to control (non-treated cells) and the LPS availability was
 817 performed with N.S: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.0001$. Bars
 818 represent the mean of 3 wells from 5 fish \pm SEM.

819

820 **Figure 7:** Respiratory burst production (A) and viability (B) in carp leucocytes after
821 treatment to Poly(I:C) ($100 \mu\text{gml}^{-1}$), MacroGard® (50 or $150 \mu\text{gml}^{-1}$) and MHCS (50 or 150
822 μgml^{-1}) both exposed individually and in combination with Poly(I:C). Statistical analysis
823 one-way ANOVA and Tukey's *post hoc* analysis were used and the differences between
824 concentrations in comparable to control (non-treated cells) and the Poly(I:C) availability was
825 performed with N.S = not significant, *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.0001$. Bars
826 represent the mean of 3 wells from 5 fish \pm SEM.

827
828 **Figure 8:** Effect of different immunostimulant and exposure time on immune gene
829 expression in carp pronephric leucocytes. Bars represent mean of relative expression
830 normalized to housekeeping gene 40s \pm SEM of three fishes. Two ways ANOVA followed
831 by Bonferroni *post hoc* analysis used to compare each treatment to the time matched control
832 *: $p \leq 0.05$; **: $p \leq 0.01$ and ***: $p \leq 0.001$.

833 **Tables**

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835 Table1: List of used qPCR primers

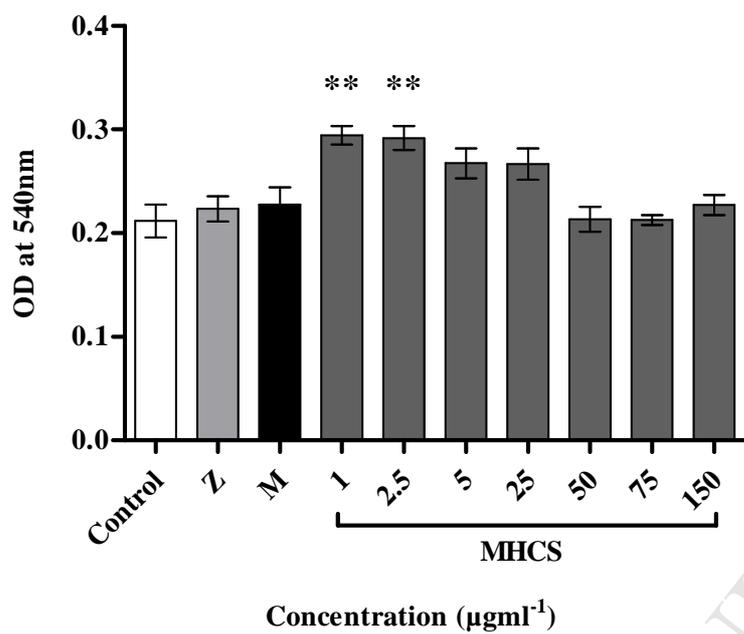
Function	Gene name	Primers sequences	Gene bank accession numbers	References
House keeping	40S	FW: 5' CCGTGGGTGACATCGTTACA 3'	AB012087	(Huttenhuis et al., 2006)
		RV: 5' TCAGGACATTGAACCTCACTGTCT 3'		
Nitric oxide production	iNOS	FW: 5' AACAGGTCTGAAAGGGAATCCA 3'	AJ242906	(Huttenhuis et al., 2006)
		RV: 5' CATTATCTCTCATGTCCAGAGTCTTCT 3'		
Pro-inflammatory cytokines	IL1 β	FW: 5' AAGGAGGCCAGTGGCTCTGT 3'	AJ245635	(Falco et al., 2012)
		RV: 5' CCTGAAGAAGAGGAGGCTGTCA 3'		
	TNF α 1	FW: 5' GAGCTTCACGAGGACTAATAGACAGT 3'	AJ311800.2	(Falco et al., 2012)
		RV: 5' CTGCGGTAAGGGCAGCAATC 3'		
	TNF α 2	FW: 5' CGGCACGAGGAGAAACCGAGC 3'	AJ311801.2	(Falco et al., 2012)
		RV: 5' CATCGTTGTGTCTGTTAGTAAGTTC 3'		
Anti-viral cytokines	IFN α 2	FW: 5' GATGAAGGTGCCATTTCCAAG 3'	AB376667	(Adamek et al., 2014)
		RV: 5' CACTGTCGTTAGGTCCATTGCTC3'		

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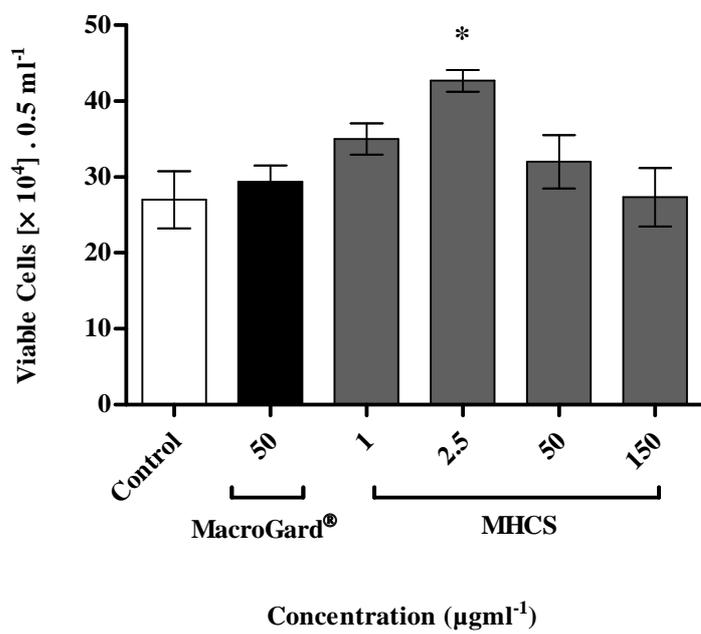
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839 **Figure 1**
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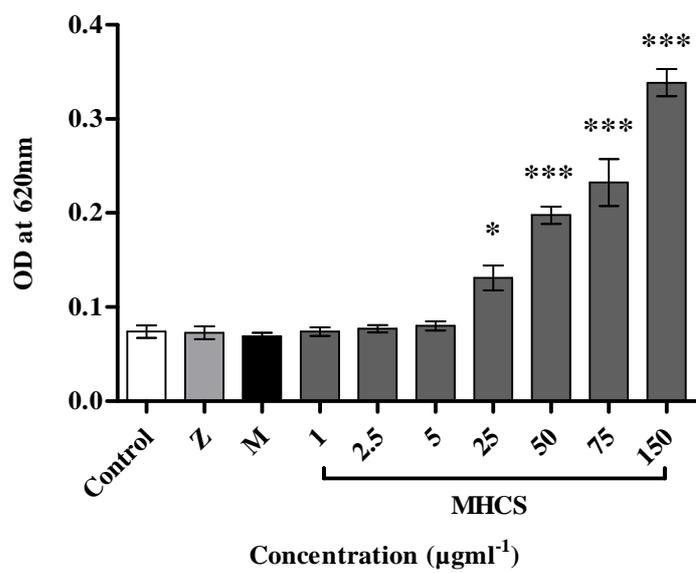
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843 **Figure 2**
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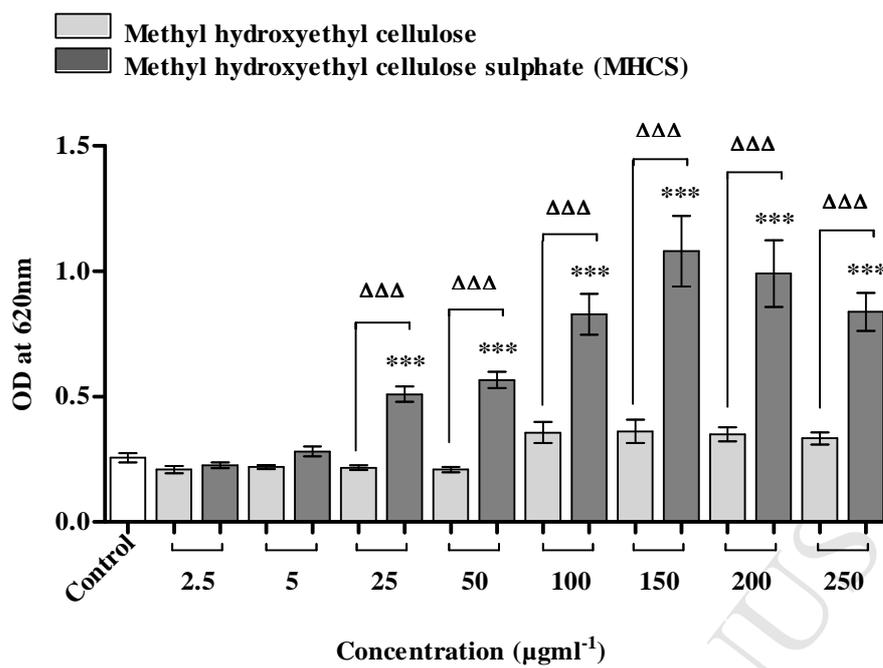
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848 **Figure 3**
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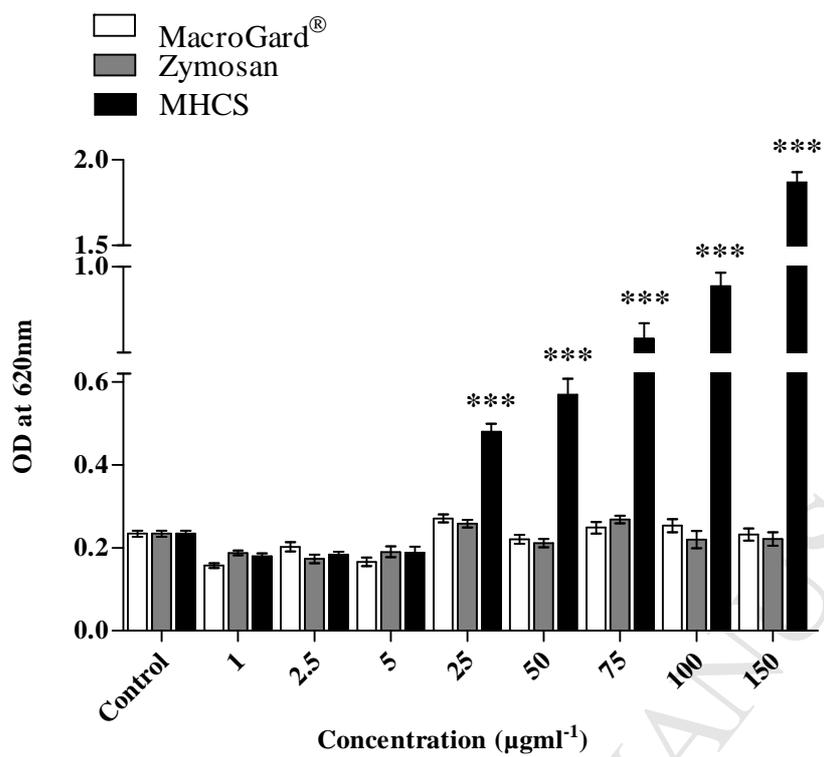
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853 **Figure 4**
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872 **Figure 7**

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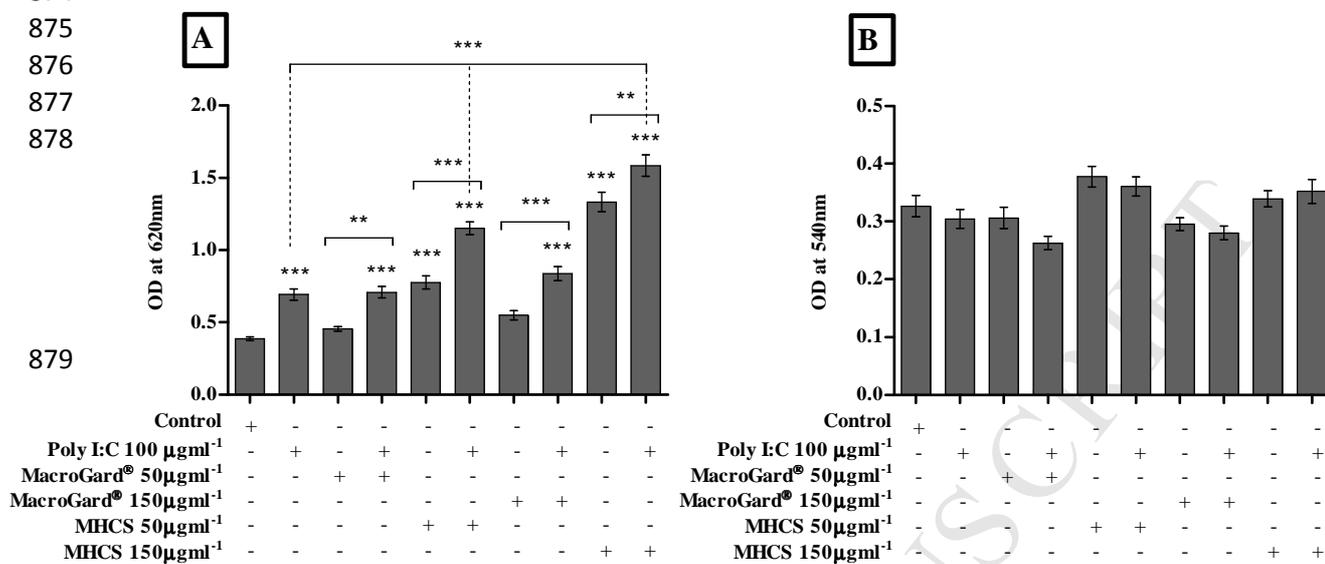
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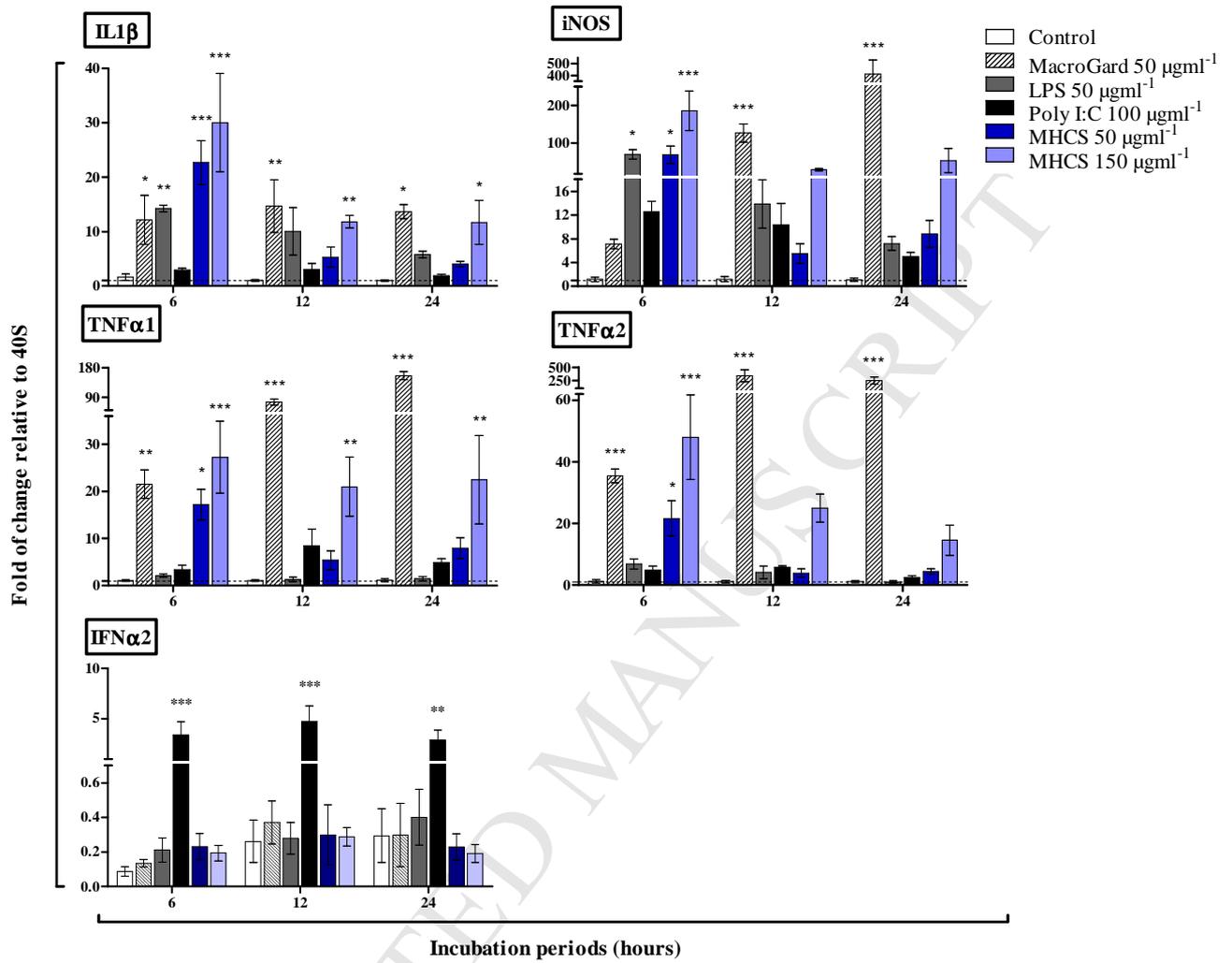
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883 **Figure 8**
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887 Colour is required for this figure in print

888 *In vitro investigations on the effects of semi-synthetic, sulphated carbohydrates on the*
 889 *immune status of common carp (Cyprinus carpio).*
 890

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892

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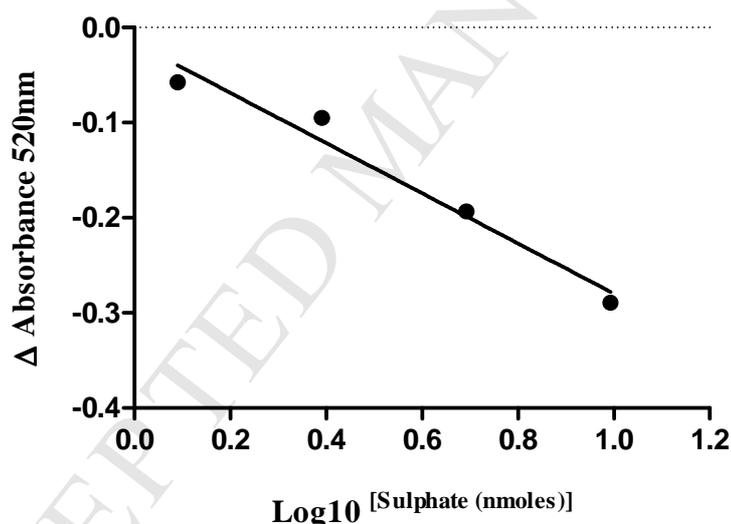
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 901 d.hoole@keele.ac.uk

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SUPPLEMENTARY DATA

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907 **Supplementary Figure 1:** Standard curve for dextran sulphate using the sulphate determination
 908 assay, employing sodium rhodizonate, as described by Terho and Hartiala, *Anal. Biochem.* 1971,
 909 41(2):471-6. $y = 0.2637x - 0.0158$; $R^2 = 0.9674$.

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911 The overall sulphation level for methyl hydroxyethyl cellulose sulphate was
 912 determined by the method of Terho and Hartiala (*Anal. Biochem.* 1971, 41(2):471-6).
 913 Briefly, dextran sulphate, of a predetermined degree of sulphation, was hydrolysed in 1 M
 914 HCl (100°C for 2 hours) prior to lyophilisation. The dry product was reconstituted in sterile
 915 water (1 mgml⁻¹) and serial dilutions performed (calibration curve) before the addition of 0.1
 916 M CH₃CO₂H, 50 uM BaCl₂, 0.8 mM NaHCO₃, 0.14 mM sodium rhodizonate and 3.4 mM L-
 917 (+)-ascorbic acid. The solution was incubated for 10 min at 20°C (in darkness) to allow
 918 colour to develop. The absorbance of the solution was ascertained at $\lambda_{\text{abs}} = 520$ nm. The assay

919 was repeated for methyl hydroxyethyl cellulose sulphate and the mass of sulphate per gram of
920 polysaccharide calculated from the dextran sulphate calibrant.

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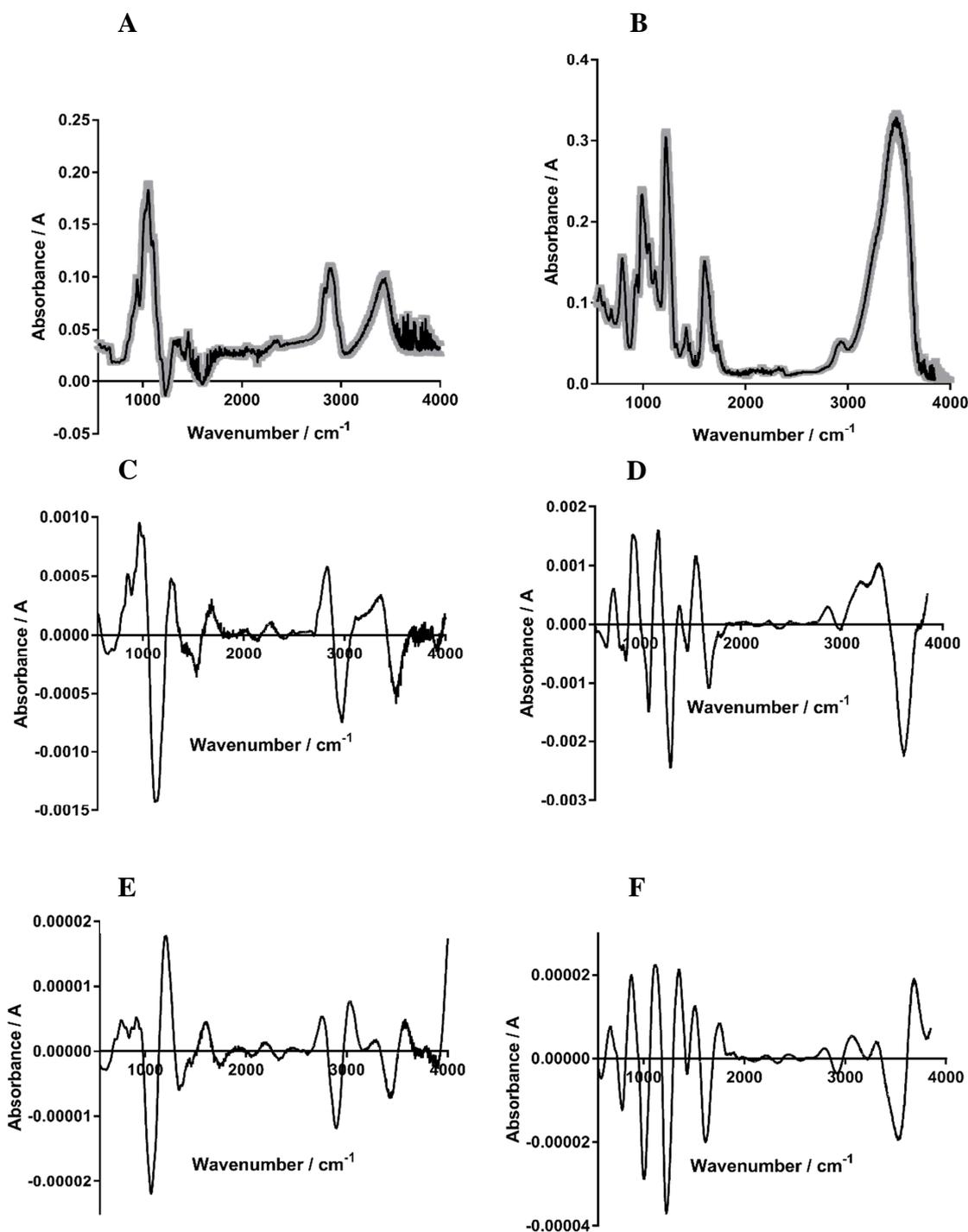
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947 **Supplementary Figure 2:** FTIR-ATR spectra for both the unsulphated (A) and sulphated (B)
948 methyl hydroxyethyl cellulose polysaccharides within the 400-4000 cm^{-1} spectral region.
949 First ((C) and (D)) and second derivative ((E) and (F)) curves are shown, respectively.

950 Attenuated total reflectance FTIR spectra were recorded for the MHCS carbohydrate
951 and the precursor using a Nicolet iS5 IR-TF (Thermo Fisher) spectrometer scanning in the
952 4000–400 cm^{-1} region with a spectral resolution of 2 cm^{-1} over 32 scans. A background air
953 spectrum was obtained and subtracted from all spectra. All carbohydrate spectra were
954 recorded using ThermoFisher Omnic software. First and second derivatives of all spectral
955 data for the precursor and modified polysaccharide were plotted and overlaid using Prism
956 software (GraphPad Software, Inc.).

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